Molecular and Functional Properties of a Calpain Activator Protein Specific for μ-Isoforms*

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A natural calpain activator protein has been isolated from bovine brain and characterized in its properties and molecular structure. The protein is a homodimer with a molecular mass of about 30 kDa and results in being almost identical to UK114 goat liver protein. Significant similarities with mouse HR12 protein were also observed, whereas a lower degree of similarity was found with a family of heat-responsive proteins named YJGF and YABJ from *Haemophilus influenzae* and *Bacillus subtilis*, respectively.

The brain activator expresses a strict specificity for the μ-calpain isoform, being completely ineffective on the m-calpain form. As expected, also UK114 was found to possess calpain-activating properties, indistinguishable from those of bovine brain activator. A protein showing the same calpain-activating activity has been also isolated from human red cells, indicating that this factor is widely expressed. All these activators are efficient on μ-calpain independently from the source of the proteinase.

The high degree of specificity of the calpain activator for a single calpain isoform may be relevant for the understanding of sophisticated intracellular mechanisms underlying intracellular proteolysis. These data are indicating the existence of a new component of the Ca^{2+}-dependent proteolytic system, constituted of members of a chaperonin-like protein family and capable of promoting intracellular calpain activation.

Calpains are a family of dimeric proteinases all characterized by an absolute dependence on Ca^{2+} (1–7). In the absence of this metal ion, calpains are stabilized in an inactive conformational state, by inter- and intramolecular constraints (8, 9). Binding of Ca^{2+} to the proteinase molecules produces both dissociation of the heterodimers (10) and conformational changes of the 80-kDa catalytic subunits, triggering the enzyme activation that is completed by an autoproteolytic event (11, 12). The concentrations of Ca^{2+} inducing the conformational changes required for the activation of both μ- and m-calpain are at least one order of magnitude higher than the actual concentrations of this metal ion in cells. Experiments designed to identify possible mechanisms effective in reducing the calcium requirement of calpains have demonstrated that association of the proteinase to phospholipid vesicles (12) or to nuclei (13) are effective in increasing its affinity for Ca^{2+}. A more relevant physiological significance is represented by a

calpain activator protein recently identified in human red blood cells (14) and in rat skeletal muscle (15). This protein factor, which is significantly effective in reducing the requirement of the proteinase for calcium ions, binds Ca^{2+} with high affinity (10) and associates to the particulate fraction of the cells; in fact, it is recovered in the soluble fraction only when cell lysis is performed by a medium containing metal chelators.

Furthermore, the activator-Ca^{2+} complex interacts with calpain and thereby induces those conformational changes required to trigger the activation process of the proteinase. On the basis of these results, the protein factor could be visualized as a physiological mean to obtain a site-directed activation of calpain elicited in response to an increased intracellular concentration of free Ca^{2+}.

In this study we report the purification of a bovine brain calpain activator. Taking advantage of new strategies for the purification procedure, we have obtained sufficient amounts of protein to establish its amino acid sequence. The primary structure of the rat brain calpain activator is almost completely identical to that of a known protein called UK114 (16), similar to that of a 23-kDa protein from rat liver (17), corrected as reported by Ceciliani *et al.* (16) and to that of HR12 heat-shock YABJ and YJGF proteins (SwissProt sequence data base). The calpain activator is highly selective for the μ-isoforms of the proteinase and does not reveal any apparent species specificity.

EXPERIMENTAL PROCEDURES

Superose 12 and Source 15 Q columns were purchased from Amersham Pharmacia Biotech. Purified UK114 (10) was kindly provided by Dr. Severino Ronchi, Istituto di Fisiologia Veterinaria e Biochimica, University of Milan, Milan, Italy.

Calpain Activator Purification—Freshly collected bovine brain was suspended in 4 volumes of 50 mM sodium acetate buffer, pH 5.8, containing 1 mM EDTA and 1 mM β-mercaptoethanol and disrupted by using Potter-Elvehjem homogenizer. Cells were lysed by sonication (6 bursts of 10 s each), and the particulate material was removed by centrifugation. The pH of the supernatant was adjusted to 5.8, and the sample was heated at 90 °C for 3 min and centrifuged, and the clear supernatant was collected. The proteins were concentrated by adding ammonium sulfate at the 80% concentration, suspended in 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA (Buffer A), dialyzed against the same buffer, then submitted to ion exchange chromatography on the Source 15 Q column (1.6 × 5 cm) equilibrated in buffer A. The unabsorbed proteins, also containing the calpain activator, were collected and applied to a butyl-agarose column equilibrated in Buffer A. The unretained material, containing calpain activator activity, was collected and submitted to gel chromatography on the Superose 12 column (1.8 × 50 cm) equilibrated in buffer A.

Calpain activator from rat skeletal muscle was purified from 120 g of fresh tissue, suspended in 10 volumes of cold 0.25 M sucrose containing 0.1 mM EDTA and 1 mM β-mercaptoethanol and homogenized in a tissue grinder. All purification steps were carried out as reported above for bovine brain activator. The activator protein was also isolated from human erythrocytes. Erythrocytes were deprived of leukocytes and platelets and washed three times with 10 mM sodium phosphate solution containing 0.15 M NaCl. Cells were then packed and lysed with 5 volumes of 50 mM sodium borate, pH 8.3, containing 1 mM EDTA. The membranes were discarded by centrifugation and the clear supernatant
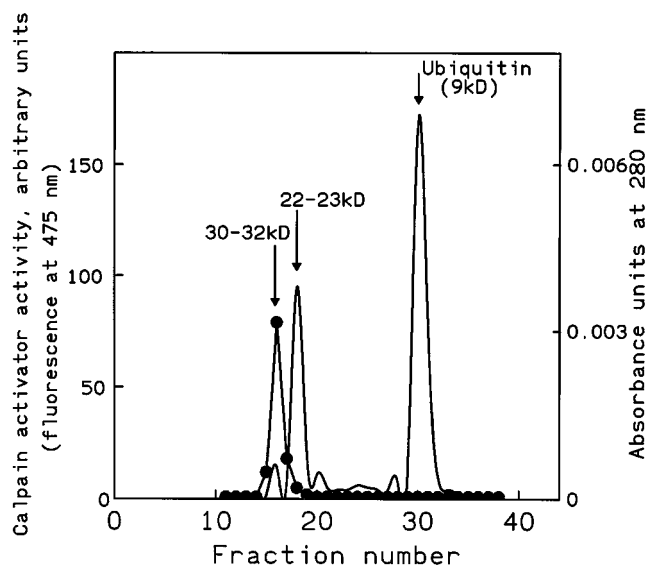
FIG. 1. **Separation of bovine brain calpain activator by gel filtration on a Superose 12 column.** Bovine brain calpain activator obtained following the hydrophobic chromatographic step (see "Experimental Procedures") was submitted to gel chromatography on a Superose 12 column previously equilibrated in Buffer A. The activator activity (●) was assayed using bovine erythrocyte μ-calpain in the presence of 2 μM Ca^{2+} (see "Experimental Procedures"). The continuous line indicates the absorbance at 280 nm.

was heated at 90 °C for 3 min. The human erythrocyte activator protein was further purified to homogeneity as described for bovine brain activator.

Assay of Calpain Activator Activity—The activator protein activity was assayed by adding the appropriate amounts of the activator sources to the routine calpain assay mixture containing 2 μM Ca^{2+} (18). One unit of calpain activator activity is defined as the amount causing the appearance of one unit of calpain activity in the presence of 2 μM Ca^{2+} (14).

Sequence Analysis of Bovine Brain Activator—Purified activator protein (100 pmol) was lyophilized in the presence of 0.05% SDS to avoid the irreversible denaturation and insolubilization of the protein, submitted to 16% SDS-PAGE,[1] and then transblotted on a polyvinylidene difluoride sheet. The region of membrane-containing activator protein, localized by staining with Ponceau S, was cut, washed with H_2O, saturated at 37 °C for 30 min with 0.5% polyvinylpyrrolidone, and then minced (19). The fragments were suspended on 0.6 ml of 50 mM sodium borate buffer, pH 8.5, containing 5% CH_3CN and 1 μg of trypsin and incubated at 37 °C for 18 h. After digestion, the soluble material was lyophilized, suspended in 50 μl of H_2O, and the pH was acidified by addition of 0.1% trifluoroacetic acid. The sample was submitted to chromatography on a C-18 column (1 × 100 mm) equilibrated with distilled water containing 0.1% trifluoroacetic acid. Absorbed peptides were eluted with a linear gradient of CH_3CN (from 0 to 70%) and fractions of 30 μl were collected. The fractions containing the tryptic peptides were directly loaded on the Beckman LF3000 protein sequencer to determine primary structure.

A comparison of primary structure of rat brain activator and other protein sequences was done using the SwissProt sequence data base. Alignment of the sequences of brain activator and goat liver UK114 was performed using the PALIGN program. The alignment of the amino acid sequence of the calpain activator protein with a rat liver 23-kDa protein (16), HR12 (HR12 MOUSE), YJGF (YJGF HAEIN), and YABJ (YABJ BACSU) proteins was obtained using the CLUSTAL program.

Purification of μ- and m-Calpain from Different Sources—μ-Calpain and m-calpain from rat brain were purified as reported (20), and those from skeletal muscle were obtained as described previously (21). Human and bovine erythrocyte μ-calpain and m-calpain were purified as reported (10).

Assay of μ- and m-Calpain Activity—Calpains were routinely assayed as previously reported (18).

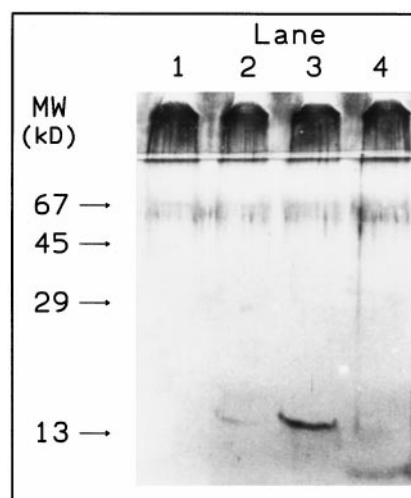[1] The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

FIG. 2. **SDS-PAGE of bovine brain activator.** A sample (200 μl) of fractions (from 13 to 16, see Fig. 1) containing activator activity obtained by Superose 12 column chromatography was lyophilized and suspended into 0.05 ml of 0.1 M Tris/HCl, pH 8.8, containing 2% 2-mercaptoethanol and 2% SDS and submitted to 16% SDS-PAGE. At the end of the electrophoretic run, the protein bands were identified by silver staining. The *arrows* indicate the molecular mass of standard proteins (bovine serum albumin, 67 kDa; chicken egg albumin, 45 kDa; carbonic anhydrase, 29 kDa; and cytochrome *c*, 13 kDa); *lanes 1–4* refer to fractions from 13 to 16 of the chromatography shown in Fig. 1.
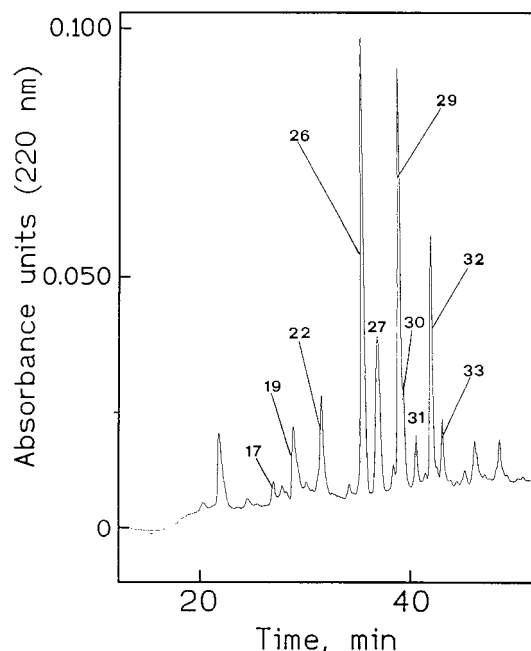
FIG. 3. **Separation of tryptic peptides of bovine brain calpain activator.** Bovine brain activator, obtained from 10 chromatographic steps as shown in Fig. 1, was submitted to SDS-PAGE (see Fig. 2) and then the proteins were transblotted on a polyvinylidene difluoride sheet (see "Experimental Procedures"). Protein bands were stained with Ponceau S, and the bands with a mobility corresponding to a mass of 14 kDa were cut and destained. The bound protein was solubilized by digestion with 1 μg of trypsin. The peptides were separated by reverse-phase chromatography on a C-18 column. Each number refers to the peptide used for the determination of the activator protein sequence.

RESULTS

Following ion exchange chromatography of the heat-treated bovine brain homogenate on Source Q resin, which binds calpastatin with high efficiency, the protein factor is recovered in the washing material. When tested for its activity, it is found to

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         1                        25                                    50
C.A.  MSSLVRKIISTAKAPAAIGPYSQAVLVDRTIYISGQLGMDPAAGQLVPGG
UK114 MSSLVRRIISTAKAPAAIGPYSQAVLVDRTIYISGQLGMDPASGQLVPGG
        17      19              29                      30
```
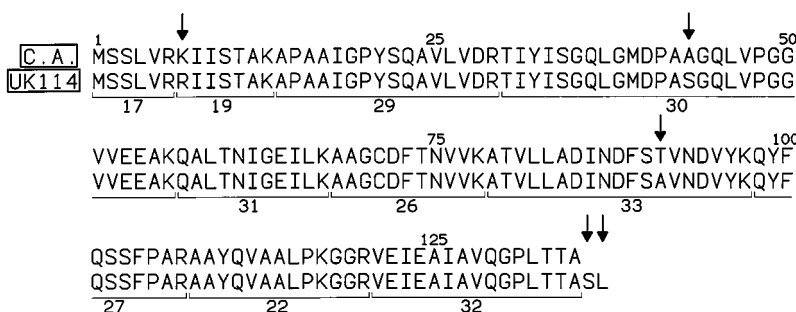
```
                              75                          100
VVEEAKQALTNIGEILKAAGCDFTNVVKATVLLADINDFSTVNDVYKQYF
VVEEAKQALTNIGEILKAAGCDFTNVVKATVLLADINDFSAVNDVYKQYF
           31              26                  33
```

```
                        125
QSSFPARAAYQVAALPKGGRVEIEAIAVQGPLTTA
QSSFPARAAYQVAALPKGGRVEIEAIAVQGPLTTASL
    27              22              32
```

FIG. 4. **Amino acid sequence of calpain activator and its similarity to that of the UK114 protein.** Peptides, isolated as shown in Fig. 3, were collected, and their amino acid sequences were determined automatically with a Beckman LF 3000 protein sequencer. The calpain activator (*C. A.*) amino acid sequence is compared with that of the UK114 protein. The *arrows* indicate differences between the two sequences.
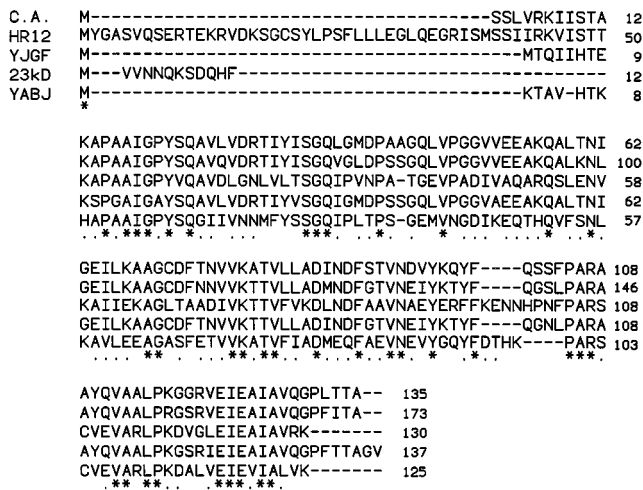
```
C.A.  M-------------------------------------SSLVRKIISTA  12
HR12  MYGASVQSERTEKRVDKSGCSYLPSFLLLEGLQEGRISMSSIIRKVISTT  50
YJGF  M-----------------------------------------MTQIIHTE   9
23kD  M---VVNNQKSDQHF-----------------------------------  12
YABJ  M----------------------------------------KTAV-HTK    8
      *

KAPAAIGPYSQAVLVDRTIYISGQLGMDPAAGQLVPGGVVEEAKQALTNI   62
KAPAAIGPYSQAVQVDRTIYISGQVGLDPSSGQLVPGGVVEEAKQALKNL  100
KAPAAIGPYVQAVDLGNLVLTSGQIPVNPA-TGEVPADIVAQARQSLENV   58
KSPGAIGAYSQAVLVDRTIYVSGQIGMDPSSGQLVPGGVAEEAKQALTNI   62
HAPAAIGPYSQGIIVNNMFYSSGQIPLTPS-GEMVNGDIKEQTHQVFSNL   57
..*.***.* *..  ..    ***. ..*.  *  ... ...* ..*.

GEILKAAGCDFTNVVKATVLLADINDFSTVNDVYKQYF----QSSFPARA  108
GEILKAAGCDFNNVVKTTVLLADMNDFGTVNEIYKTYF----QGSLPARA  146
KAIIEKAGLTAADIVKTTVFVKDLNDFAAVNAEYERFFKENNHPNFPARS  108
GEILKAAGCDFTNVVKTTVLLADINDFGTVNEIYKTYF----QGNLPARA  108
KAVLEEAGASFETVVKATVFIADMEQFAEVNEVYGQYFDTHK----PARS  103
 .... **  ...**.**.. *...*..**.  *  ...    ***.

AYQVAALPKGGRVEIEAIAVQGPLTTA--  135
AYQVAALPRGSRVEIEAIAVQGPFITA--  173
CVEVARLPKDVGLEIEAIAVRK------  130
AYQVAALPKGSRIEIEAIAVQGPFTTAGV  137
CVEVARLPKDALVEIEIVIALVK------  125
 .** **.. .***.**.
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FIG. 5. **Structural similarities among bovine brain calpain activator (*C. A.*), HR12, 23-kDa rat liver protein, and YJGF and YABJ proteins.** The amino acid sequences of HR12, YJGF, and YABJ proteins were taken from the SwissProt data base. The sequence of the 23-kDa protein was taken from the work of Ceciliani *et al.* (16). *, indicate a perfectly conserved residue; ·, indicates a well conserved residue.

FIG. 6. **Activity of UK114 protein on μ-calpain.** A sample of UK114 protein (20 μg) dissolved in water was submitted to Superose 12 chromatography in the same conditions used for brain activator (see Fig. 1 and "Experimental Procedures"). Aliquots of the eluted fractions (30 μl) were assayed for calpain activator activity using bovine erythrocyte μ-calpain (see "Experimental Procedures").

promote activation of calpain at a concentration of calcium ineffective to promote activity of the native proteinase. The solution is then filtered through a column of butyl-agarose, which removes the residual traces of calpastatin activity and other contaminating proteins. Finally, the solution containing calpain activator activity is submitted to gel chromatography on a Superose 12 column. As shown in Fig. 1, three major protein peaks are eluted: the first one showing an approximate molecular mass of 30 kDa (emerging immediately before carbonic anhydrase, 29 kDa); a second one with a mass of approximately 22 kDa; and a third one with a mass below 10 kDa and identified as ubiquitin by its N-terminal amino acid sequence (data not shown). Only the first peak contains the calpain activator activity. All fractions under this peak are collected and submitted to SDS-PAGE (Fig. 2). A single protein band is detected with a mobility corresponding to a molecular mass of approximately 15 kDa, indicating that in the native active form the activator is present in a dimeric structure.

To further characterize the protein, the material recovered from ten separate chromatographic analyses, each one corresponding to that shown in Fig. 1, is loaded on a SDS-PAGE and transblotted on a polyvinylidene difluoride sheet. The position of the single protein band is identified by staining with Ponceau S, cut, and destained as described under "Experimental Procedures." The protein is then solubilized by digestion with trypsin, and the peptides mixture separated on a C-18 reverse-phase chromatography (Fig. 3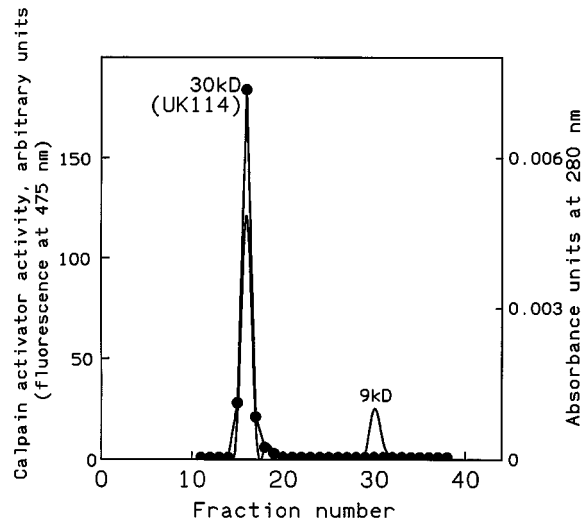). The peptides are eluted by a linear gradient of acetonitrile (from 0 to 70%) and each peptide is separately collected, sequenced, and analyzed for its similarity with known proteins using SwissProt sequence data base (program FASTA3). All peptides show a sequence almost completely identical to the corresponding tryptic peptides from a known protein named UK114. The sequences are then aligned following the primary structure of UK114 protein, and the results are shown in Fig. 4. The differences between the two amino acid sequences are confined at residues 7, 43, and 91 at the C-terminal end. The calpain activator protein also shows structural similarities with a 23-kDa protein from rat liver (16) with the mouse heat-responsive protein HR12, and with two families of proteins, members of which (YJGF and YABJ) are reported in Fig. 5. The similarity of this protein factor with heat-shock proteins is very suggestive and might indicate a "chaperonin-like function."

For comparison we have analyzed the functional properties of UK114 on calpain activity, due to the almost complete identity between the calpain activator protein and UK114. A sample of UK114 protein is submitted to Superose 12 column chromatography in the conditions used for the isolation of the brain calpain activator (Fig. 6). Two protein peaks are separated; the major is eluted in an identical volume to that of the calpain activator, the second one in an elution volume corresponding to ubiquitin. The first peak is found to possess a potent activating effect on calpain, almost indistinguishable

Bovine Brain Calpain Activator

TABLE I

Effect of bovine brain calpain activator on the Ca^{2+} requirement and on the V$_{max}$ of μ- and m-calpains isolated from different sources

Ca^{2+} requirement was determined assaying calpain activity at increasing concentration of Ca^{2+} in the absence or presence of calpain activator in an amount causing the maximal effect (see Fig. 7).

Calpain		Ca^{2+} requirement[a]		V$_{max}$[b]
Source	Isoform	−Activator	+Activator	
		μM		
Rat brain	μ	12 ± 2	0.45 ± 0.05	1.04
Rat skeletal muscle	μ	10 ± 1	0.40 ± 0.05	1.09
Bovine erythrocytes	μ	15 ± 3	0.52 ± 0.05	1.05
Human erythrocytes	μ	27 ± 3	0.50 ± 0.08	1.12
Rat brain	m	250 ± 30	250 ± 30	1.0
Rat skeletal muscle	m	295 ± 30	295 ± 30	1.0

[a] Expressed as the [Ca^{2+}] promoting 0.5 V$_{max}$.
[b] Expressed as the ratio between V$_{max}$ measured in the presence of the activator and V$_{max}$ measured in its absence.
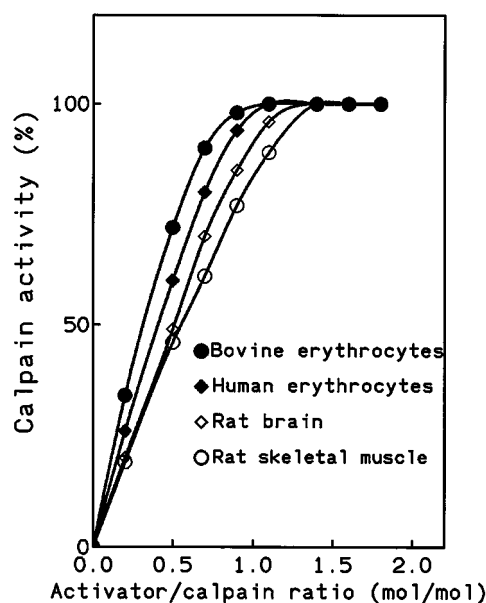

FIG. 7. **Effect of rat brain activator on the catalytic activity of μ-calpain isolated from different sources.** μ-Calpain was purified from human or bovine erythrocytes and rat brain or skeletal muscle as described under "Experimental Procedures." Calpains (0.01 nmol) were incubated with increasing amounts of activator at the indicated molar ratio. Calpain activity was assayed in the presence of 2 μM Ca^{2+} and is expressed at the percentage of total proteolytic activity measured in the presence of 1 mM Ca^{2+}.

from that observed with the authentic bovine brain calpain activator.

As shown in Table I, addition of the bovine brain activator to μ-calpains, isolated from different sources, promotes a more than 10-fold decrease in the Ca^{2+} requirement of the proteinases without any modification of their V$_{max}$. On the contrary, the activator is ineffective on rat brain or skeletal muscle m-calpain.

The activator effect is dose-dependent (Fig. 7) and reaches a maximum on activator/calpain molar ratio of approximately 1 to 1. Rat skeletal muscle μ-calpain shows a slightly lower affinity for the activator and requires a 1.1–1.2 factor excess.

In Table II, the properties of calpain activators from human erythrocyte and goat liver sources are compared with those of the bovine brain protein factor. The three activators are equally efficient in promoting expression of catalytic activity of homologous and heterologous μ-calpains. All of these protein factors are ineffective on m-calpains.

Taken together, these findings indicate that the μ-calpain

TABLE II

Isozyme and tissue specificity of calpain activator

Calpain activator effect is expressed as the percentage of calpain activity measured in the presence of 2 μM Ca^{2+} and equimolar amounts of activators. As 100% was taken the calpain activity assayed in the presence of 1 mM Ca^{2+}. The effect of the activator on m-calpains was determined assaying the enzyme activity at 20, 50, and 100 μM and 1 mM Ca^{2+} with or without equimolar amounts of the indicated protein factors.

Calpain		Calpain activator from		
Source	Isozyme	Bovine brain	Human erythrocytes	Goat liver UK114
		%		
Rat brain	μ	88	91	91
Rat skeletal muscle	μ	82	85	86
Bovine erythrocyte	μ	104	107	106
Human erythrocyte	μ	97	95	98
Rat brain	m	ND[a]	ND	ND
Rat skeletal muscle	m	ND	ND	ND

[a] ND, not detectable.

activator protein is not a characteristic of brain tissue, and it is probably widely distributed.

DISCUSSION

It is generally recognized that the soluble calcium-dependent proteolytic system is composed by one or more proteinases (calpains) and by their natural inhibitor, calpastatin (1–6, 22–25). In the past years, we have observed that a possible third component of this system, effective in reducing the Ca^{2+} requirement of calpains and thereby in promoting activation of these proteinases, is also present in different human and rat tissues (14, 15). Its molecular characterization and degree of identity with other proteins have never been defined, probably due to difficulties in the preparation procedures and in the concentration of the samples as well as in the chromatographic analyses. This protein, in fact, binds to many inert supports or membranes particularly to those employing hydrophobic materials. These difficulties have been overcome by the use of new purification strategies; on the basis of which it was possible to obtain a sufficient amount of pure calpain activator to determine the primary structure.

The molecular mass resulted in being slightly higher than that of cytochrome *c*, approximately 15 kDa in denaturing conditions and around 30 kDa in native conditions, indicating that the native active form is a dimer. Sequence analysis has revealed its almost complete identity with a previously characterized protein isolated from the acid-soluble material of goat liver and called UK114 (16). In addition, the bovine brain calpain activator shows structural similarities with a 23-kDa protein from goat liver (16, 17), a high degree of similarity with the mouse heat-responsive protein HR12, or with heat-shock proteins (YJGF and YABJ), suggesting that the mechanism of action may also include a chaperonin-like activity directed at promoting conformational changes of target proteins. This hypothesis is in agreement with the previously described functional properties of the calpain activator and consistent with a sequential mechanism of activation involving at first an interaction of the Ca^{2+}-activator complex with calpain, followed by a conformational change of the proteinase, resulting in its activation at very low [Ca^{2+}].

Since the activator interacts with intracellular membrane structures upon binding of Ca^{2+} (data not shown), it could also be suggested that the overall mechanism consists of promoting a site-directed activation of calpain and thereby a site-directed specificity for digestion of protein substrates. This model can be considered to operate with a high degree of specificity in different cells in which the soluble calcium-dependent proteolytic system requires a sophisticated regulatory efficiency. However,

we have observed that the calpain activator isolated from rat skeletal muscle, although showing a similar activating mechanism, displays different specificity in activating m-calpain (26). The presence of multiple activator forms, together with the observed structural similarities among the protein factor and other protein molecules, suggests the existence of a family of calpain-activating proteins with specific properties operating in various tissues in response to different stimuli. As revealed by the Garnier analysis method (27), the calpain activator and the proteins showing a high degree of similarity with this factor contain a central region rich in helical conformation-preferring residues (residues 52–72 in the calpain activator protein), suggesting the existence of common structural features that could be involved in similar intracellular functions.

Furthermore, these results represent the first demonstration for an intracellular function of UK114 protein related to the modulation of the soluble calcium-dependent proteolytic system and thereby stressing the role of the calcium-mediated proteolysis in cell functions.

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